RESEARCH LETTER

Cultural conditions required for the induction of an adaptive acid-tolerance response (ATR) in *Sinorhizobium meliloti* and the question as to whether or not the ATR helps rhizobia improve their symbiosis with alfalfa at low pH

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Received 24 September 2009; accepted 20 October 2009.
Final version published online 2 December 2009.

DOI:10.1111/j.1574-6968.2009.01846.x

Editor: Yaacov Okon

Keywords
rhizobia; acidity; ATR; nodulation; competitiveness.

Abstract

Sinorhizobium meliloti associates with *Medicago* and *Melilotus* species to develop nitrogen-fixing symbioses. The agricultural relevance of these associations, the worldwide distribution of acid soils, and the remarkable acid sensitivity of the microsymbiont have all stimulated research on the responses of the symbionts to acid environments. We show here that an adaptive acid-tolerance response (ATR) can be induced in *S. meliloti*, as shown previously for *Sinorhizobium medicae*, when the bacteria are grown in batch cultures at the slightly acid pH of 6.1. In marked contrast, no increased tolerance to hydrogen ions is obtained if rhizobia are grown in a chemostat under continuous cultivation at the same pH. The adaptive ATR appears as a complex process triggered by an increased hydrogen-ion concentration, but operative only if other – as yet unknown – concomitant factors that depend on the culture conditions are present (although not provided under continuous cultivation). Although the stability of the ATR and its influence on acid tolerance has been characterized in rhizobia, no data have been available on the effect of the adapted state on symbiosis. Coinoculation experiments showed that acid-adapted indicator rhizobia (ATR+) were present in $> 90\%$ of the nodules when nodulation was performed at pH 5.6, representing a $> 30\%$ increase in occupancy compared with a control test. We show that the ATR represents a clear advantage in competing for nodulation at low pH. It is not yet clear whether such an effect results from an improved performance in the acid environment during preinfection, an enhanced ability to initiate infections, or both conditions. The practical use of ATR+ rhizobia will depend on validation experiments with soil microcosms and on field testing, as well as on the possibility of preserving the physiology of ATR+ bacteria in inoculant formulations.

Introduction

Biological nitrogen fixation mediated by the legume–rhizobia symbioses is important for world agriculture. The productivity of legume crops is significantly affected by soil acidity. The low pH of soils may markedly reduce the productivity of legumes mainly because of the detrimental effects of hydrogen ions on the rhizobia and on their symbiosis with legumes (Munns, 1968; O'Hara *et al.*, 1989). *Sinorhizobium meliloti* and *Sinorhizobium medicae* – the symbionts of *Medicago*, *Melilotus* and *Trigonella* spp. – have been shown to be extremely sensitive to low pH (Glenn & Dilworth, 1994), with their growth slowing down and even stopping at pH 5.5 or below (Howieson *et al.*, 1992; Reeve

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et al., 1993). Acid tolerance in rhizobia has been considered a key phenotypic characteristic in that it enables the bacteria to perform well under the otherwise restrictive conditions of excessive acidity (Howieson *et al.*, 1988). The screening for acid-tolerant isolates that can colonize and/or persist in acidic soils thus gave rise to novel strains with enhanced survival and/or symbiosis under moderately acid conditions (Thornton & Davey, 1984; Richardson & Simpson, 1989; Graham *et al.*, 1994; Del Papa *et al.*, 1999, 2003; Segundo *et al.*, 1999). Complementary to this approach, the identification of the genetic determinants of acid tolerance in *S. meliloti* has also been considered a key strategy in the attempt to manipulate and improve bacterial survival and symbiosis at low pH. At the moment, however, there are few sinorhizobial genes that have been identified as genetic markers for the acid-tolerant phenotype – i.e. *act* genes (Goss *et al.*, 1990; Tiwari *et al.*, 1992, 1996a, b; Kiss *et al.*, 2004). In *S. medicae*, certain genes that were shown to be transcriptionally upregulated at low pH nevertheless do not appear to be essential for the growth of the bacteria under acid conditions (Reeve *et al.*, 1999). The available evidence indicates that tolerance to acidity in *Sinorhizobium* spp. is a multigenic phenotype in which the genetic determinants appear to be associated with diverse cellular functions.

Mutagenesis of sinorhizobia and studies on their tolerance to acidity helped the identification of genes that were shown to be essential for the growth at low pH such as *actA*, *actP*, *exoR*, *actR*, and *actS* (Howieson *et al.*, 1988; Tiwari *et al.*, 1992, 1996a, b; Graham *et al.*, 1994). However, studies on rhizobial tolerance to acidity in soils revealed that an 'acid-tolerant' rhizobium in laboratory cultures does not necessarily insure an outstanding survival and competition of the same rhizobia under comparable acid conditions in soil (Lowendorf & Alexander, 1983; Brockwell *et al.*, 1991). Even more uncertain is the correlation between the rhizobial ability to persist in acid soils and the capacity of these bacteria to express their symbiotic phenotype in the same acidity (Bromfield & Jones, 1980; Rice, 1982; Hartel & Alexander, 1983; Howieson *et al.*, 1988). Nonetheless, acid tolerance in artificial media is considered a positive characteristic when selecting rhizobia for the improvement of inoculant products for acid soils (Howieson & Ewing, 1986; Glenn & Dilworth, 1994).

As the pH decreases below 7.0, there is initially no effect on the mean generation time of *S. meliloti*, but further decreases in pH (usually below 6.0) lead bacteria to a rapid decrease in their growth rate within a narrow range of 0.2 U. Interestingly, while growing at a sublethal acid pH, *Rhizobium leguminosarum* bv. viciae and *S. medicae* exhibit an adaptive acid-tolerance response (ATR) that is influenced by the calcium concentration (O'Hara & Glenn, 1994; Dilworth *et al.*, 1999). The ATR is defined as the cells' resistance to an acid shock when they have been grown for a certain time at a moderately low pH. *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium, among other bacteria, exhibit an ATR when exposed to a mildly acidic pH (Foster, 1995; Davis *et al.*, 1996). Furthermore, ATR was shown to be growth-phase specific (Davis *et al.*, 1996), with different responses occurring in both logarithmic and stationary phases, and the onset requires the *de novo* synthesis of acid-shock proteins (Foster, 1991, 1993). The ATR confers cross-resistance to other stresses as well, such as heat, sodium chloride, and ethanol (Leyer & Johnson, 1993; Lou & Yousef, 1997); there is some evidence that the resistant state may be accompanied by an increased bacterial virulence (O'Driscoll *et al.*, 1996). In *S. medicae*, the two-component sensor–regulator system, *actSR*, was shown to be essential for the induction of this adaptive ATR (Glenn *et al.*, 1999).

While the basic aspects of symbiosis have been characterized extensively, further work is needed in order to increase our knowledge concerning the rhizobial ecology under suboptimal environmental conditions such as acidity. In this context, the rational manipulation of the rhizobial acid tolerance will require a detailed physiologic and functional characterization of the processes leading to the acid-tolerant state. To this end, we have established batch and continuous cultures of *S. meliloti* under controlled pH conditions and evaluated the bacterial entrance in the adaptive ATR and the symbiotic ability for nodulation. In contrast to the batch cultivation, in steady-state chemostat cultures, individual environmental parameters can be manipulated in a controlled manner and at a fixed specific growth rate. The goal of the present study was to analyze the influence of acidity and culture conditions on ATR expression in *S. meliloti*, and to focus specifically on the subsequent effect of cultivation parameters on the bacterial competitiveness for nodulation of the host plant *Medicago sativa*.

Materials and methods

Bacterial strains, culture medium, and pH control

Sinorhizobium meliloti 2011 (J. Denairé, Toulouse, France) was used in this work. For plant competition studies, *S. meliloti* 20MP6 [a green fluorescent protein (GFP) derivative of *S. meliloti* 2011] was used (Pistorio *et al.*, 2002).

Batch and continuous culture establishment

Batch cultures and nutrient-limited continuous cultures were established in Evans minimal medium (Evans *et al.*, 1970) containing 10 g L^{-1} glucose as a carbon source and 0.7 g L^{-1} ammonium chloride as a nitrogen source (the limiting growth component in the chemostat). In batch cultures, the pH was controlled by the addition of 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) or 20 mM piperazine *N*,*N*′-bis-(2-ethanesulfonic) acid (PIPES) to

keep the pH close to 6.1 or 7.0, respectively. In the continuous cultures in the chemostat, the pH was automatically monitored with a precision of ± 0.05 U and maintained at either 7.0 or 6.1 by the addition of 1 M NaOH when necessary. In the batch cultures, the rhizobia were grown at 28 °C and 250 r.p.m. in a rotary shaker up to the early log phase of growth ($OD_{600\,nm} = 0.2$). Each primary culture was inoculated to insure at least two generations of growth before exposure to severe acid shock. The continuous cultures were grown in the same Evans medium at 28 °C in a 2-L Bioflo IIe (New Brunswick Scientific Co., Edison, NJ) reactor with a working volume of 1.5 L. The dilution rate was adjusted at $0.07 \pm 0.01\ h^{-1}$. The cultures were flushed with air ($20\ L\ h^{-1}$) and the dissolved-oxygen concentration was measured continuously by means of an Ingold polarographic probe (Wilmington, MA). The cultures were considered to be under steady-state conditions when the biomass concentration and specific rate of oxygen consumption varied by $< 10\%$.

Exposure of *S. meliloti* 2011 to a severe acid shock

To investigate the occurrence of an adaptive ATR in the strain *S. meliloti* 2011, 1 mL of the bacterial culture of interest was centrifuged at 14 000 ***g*** for 5 min at room temperature and resuspended in 1 mL of fresh Evans medium at pH 4.0 and a cell density of about $2 \times 10^8\ CFU\ mL^{-1}$ (beginning of the acid shock, $t = 0$). The study was performed both with batch cultures in the early log phase of growth and with steady-state continuous cultures growing at either pH 7.0 or 6.1, as indicated. During the acid shock at pH 4.0, the rhizobial cells were incubated at 28 °C and 180 r.p.m. in a rotary shaker in order to maintain aerobic conditions. Aliquots were removed at 1-h intervals and plated on agarized Evans medium, pH 7.0, in order to count the viable cells that had survived the acid shock. The viable counts obtained were plotted on a semi-log scale and the decimal reduction time (D_{10}) – the time required for the number of cells to be reduced by a factor of 10 – was determined from the calculated slope. The D_{10} values were compared by mean of the Student *t*-test ($P \leq 0.01$).

Nodulation kinetics

The nodulation kinetics of *S. meliloti* 2011 harvested from continuous cultures established at pH 7.0 and at pH 6.1 were analyzed in plastic pouches with modified nitrogen-free Fåhraeus medium (Lodeiro *et al.*, 2000) at both pH 7.0 (20 mM PIPES) and pH 5.6 (20 mM MES). Seeds of *M. sativa* cv. Monarca, INTA, Argentina, were surface-sterilized for 10 min with 30% v/v commercial bleach (equivalent to $55\ g\ L^{-1}$ active Cl_2), followed by six washes with sterile distilled water. The seeds were then germinated on 1.5% w/v water-agar. Two-day-old seedlings were transferred to ethylene oxide-sterilized plastic growth pouches containing 10 mL of the corresponding Fåhraeus solution. Three days later, each root was inoculated with *c.* 10^5 CFU of the corresponding rhizobia by dripping 100 μL of bacterial suspension onto the root from the tip to the base. The plants were cultured in a growth chamber at 22 °C, with a photoperiod of 16 h/8 h – day/night. The number and relative location – on the primary and secondary roots – of individual nodules were then scored for the 3 weeks that followed the inoculation.

Plant tests to compare the nodulation competitiveness of ATR+ and ATR− rhizobia

Plastic pots with 70 g of sterile vermiculite were used in the competition experiments. Five 2-day-old seedlings were transferred to each plastic pot irrigated with modified nitrogen-free Fåhraeus mineral solution buffered at either pH 7.0 or 5.6. Five days later the alfalfa plants were inoculated with 50 mL of a mix containing $1 \times 10^5\ CFU\ mL^{-1}$ of *S. meliloti* 20MP6 (GFP) and $1 \times 10^5\ CFU\ mL^{-1}$ of *S. meliloti* 2011; each of them either acid-adapted (ATR+, grown in batch cuture at pH 6.1) or control (ATR−, grown in batch culture at pH 7.0), as indicated. Thirty days after the inoculation, the root nodules were excised, washed, and observed under a Leica MZ8 fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany) to detect the presence of strain *S. meliloti* 20MP6 within the nodules. Previous work from our laboratory had shown that nodule co-occupancy with two inoculated genotypes represented an event of low incidence (Lagares *et al.*, 1992). The number of nodules occupied by each rhizobia were log-normalized and analyzed by means of the Student *t*-test ($P \leq 0.05$).

Results and discussion

Acidic pH in the extracellular medium is not a sufficient condition for triggering the adaptive ATR in *S. meliloti*

As indicated above, the bacterial adaptive ATR has been largely reported in enteric bacteria as well as in rhizobia. In order to investigate the conditions that are necessary for the generation of an ATR in *S. meliloti*, rhizobia were grown in batch-culture systems and in a chemostat under continuous cultivation, both at neutrality and under moderate acidity (pH = 6.1), and the death rates of the resulting bacteria were evaluated at pH 4.0 (see Materials and methods). As previously shown for *S. medicae* WSM419 grown at pH 5.6 (Dilworth *et al.*, 1999), *S. meliloti* 2011 grown in batch cultures at pH 6.1 displayed a significantly lower death rate during the subsequent acid shock compared with rhizobia

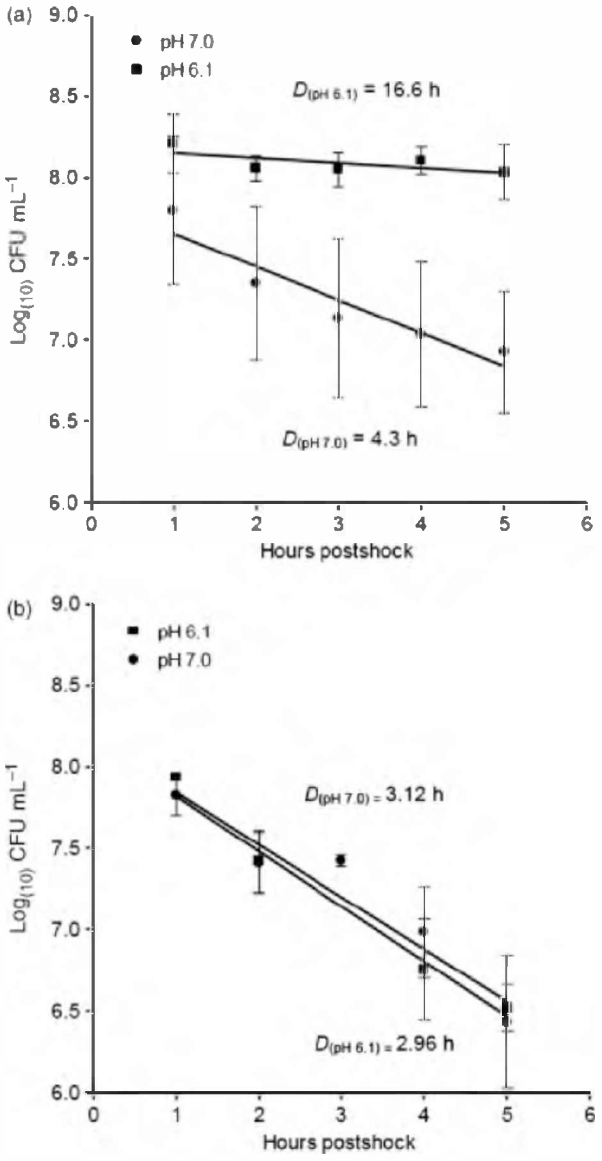

Fig. 1. Death rates of cells of *Sinorhizobium meliloti* 2011 grown in Evans minimal medium under different cultural conditions. (a) Death rates at pH 4.0 of *S. meliloti* cells previously grown in batch cultures at either pH 7.0 or 6.1. Bacterial cells from a culture in the early-log phase of growth were centrifuged and incubated in the same volume of fresh Evans medium at pH 4.0 as indicated in Materials and methods. The death rate was followed by plating appropriate dilutions of the bacterial suspension at the indicated times in agarized Evans medium. The D_{10} values corresponding to the bacteria previously grown in the acid (D = 16.6 h) and the neutral (D = 4.3 h) batch cultures are statistically different from one another ($P \leq 0.05$). (b) Death rates at pH 4.0 of *S. meliloti* cells previously grown under continuous cultivation at either pH 7.0 or 6.1. The D_{10} values were determined as indicated for the cells previously grown in the batch cultures in (a). No significant difference was observed here between the D_{10} values for the cells previously grown in the neutral and the acid continuous cultures.

that had been cultivated in batch at pH 7.0 (Fig. 1a). In these experiments, cells of *S. meliloti* 2011 were grown to mid-exponential phase ($OD_{600\ nm}$ = 0.2) at pH 7.0 or 6.1 in Evans minimal medium and resuspended in an acid-shock medium (Evans, at pH 4.0; see Materials and methods). Rhizobia that had been precultivated in batch at pH 6.1 improved their decimal reduction time (D_{10}) by a factor of 3.7 compared with rhizobia that had been grown in similar batch cultures under neutral conditions (a D_{10} of 16.6 h compared with 4.3 h, respectively).

In striking contrast, when parallel studies on survival at pH 4.0 were carried out with rhizobia harvested from the chemostat, no differences were observed between the cells collected at pH 6.1 and at pH 7.0 (Fig. 1b). Both types of rhizobia showed similar D_{10} values (*c.* 2.9 h), which were slightly lower than the D_{10} of the less-tolerant rhizobia grown in the batch culture at pH 7.0 (Fig. 1a). We need to emphasize here that the dilution rates had been kept constant during the steady states reached at pH 6.1 and 7.0 in order to avoid changes in acid tolerance that could arise from differences in the duplication time of the rhizobia growing at the two pH. The results, thus, show that the ATR induced when rhizobia grow at low pH in batch culture cannot be triggered by the same acid pH under continuous cultivation, thus indicating that exposure to acidity *per se* is an insufficient condition for evoking a shift to the transient state of increased acid tolerance.

The nodulation kinetics of rhizobia grown in the chemostat

In the previous section, we showed that cells collected from the continuous cultures have a comparable D_{10} upon subsequent severe acid shock, irrespective of the pH during cultivation. To evaluate how the same rhizobia compared in their symbiotic capabilities, we studied their nodulation kinetics after inoculation onto alfalfa plants growing in Fåhraeus medium at pH 7.0 or 5.6. Nodulation of rhizobia from the neutral chemostat was better at pH 7.0 than at pH 5.6 (Fig. 2a), in agreement with previous results obtained with cells from batch cultures (Munns, 1970). The results from this experiment also indicated that bacteria grown in the chemostat at pH 6.1 nodulate with comparable kinetics at pH 7.0 and 5.6 (Fig. 2b, black vs. gray curves). That is, rhizobia grown at pH 6.1 did not significantly modify their nodulation kinetics when changing the pH of the plant medium. In addition, bacteria from the chemostat at pH 6.1 did not reach, at neutral pH, the same total number of nodules as the rhizobia grown at pH 7.0 (black curves, Fig. 2b vs. 2a). Overall, the results indicate that while bacteria grown in the chemostat at different pH did not significantly differ in their tolerance to a severe acid shock (Fig. 1b), they

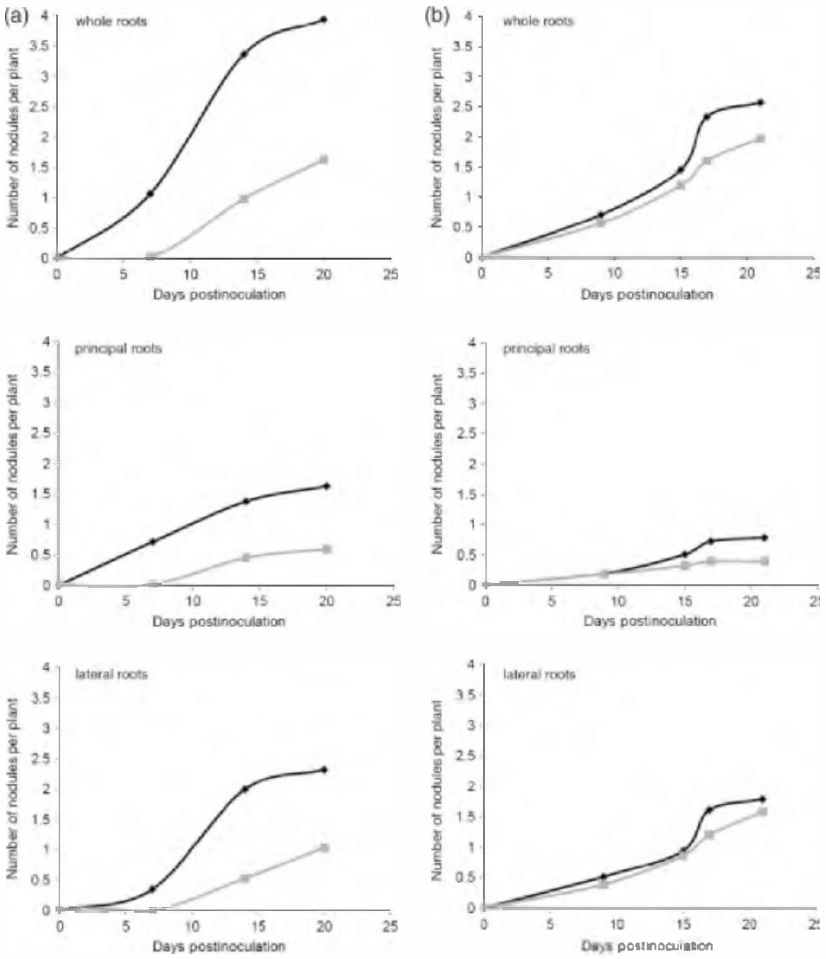

Fig. 2. Nodulation kinetics of alfalfa plants in Fåhraeus medium at different pH inoculated with *Sinorhizobium meliloti* 2011 grown in continuous culture either at neutral pH or at pH 6.1. (a) Number of root nodules/plants at different times postinoculation in alfalfa plants inoculated with rhizobia harvested from a continuous culture established at pH 7.0 (see Materials and methods). (b) Number of root nodules/plants at different times postinoculation in alfalfa plants inoculated with rhizobia harvested from a continuous culture established at pH 6.1. The black and gray lines correspond to plants in Fåhraeus solution at pH 7.0 and 5.6, respectively. Nodulation tests were performed in plastic pouches as indicated in Materials and methods with inocula of approximately 10^5 CFU per plant. Nodules in the different topologies of the root were scored by visual inspection during the first 3 weeks postinoculation.

behaved differently when inoculated on *M. sativa* at pH 7.0 (Fig. 2a and b).

Nodulation of rhizobia that have entered the adaptive ATR: competitiveness for nodule occupancy

Thus far, we have seen that *S. meliloti* 2011 is able to increase its tolerance to a severe acid shock when the bacteria have been previously cultivated in batch at a moderately acidic pH. In order to explore whether the adaptive ATR represents a positive trait for nodulation at low pH as well, we compared the relative ability of adapted (ATR+) and nonadapted (ATR−) rhizobia to form nodules when they were coinoculated in comparable numbers on alfalfa plants at different pH. Wild-type *S. meliloti* 2011 were used as control rhizobia cultivated at pH 7.0, and the isogenic GFP derivative 20MP6 (Pistorio *et al.*, 2002) as ATR+ coinoculant competitors (Fig. 3a). The results clearly showed a marked dominance of ATR+ rhizobia within the nodules when the nodulation test was performed under acid conditions (> 90% occupancy), thus strongly suggesting that the adaptive ATR operates as a significant positive trait, enabling competition for the infection of the host root at low pH. Figure 3b shows a control assay where both the *S. meliloti* 2011 ATR− and its isogenic derivative 20MP6 ATR+ were cultivated at the same pH (either neutral or acid) and then coinoculated onto plants growing either on neutral or acid Fåhraeus medium. The remarkable competitiveness of the acid-adapted rhizobia at low pH is most probably a consequence of better performance during the preinfection before the bacteria penetrate the root. The increased tolerance to acidity of ATR+ rhizobia would likely make them more proficient under the acid stress in sustaining those energy-requiring cellular activities that are necessary for survival and to enter into symbiosis. Nonetheless, because in other bacteria the adaptive ATR has been shown to provide cross-protection against different, unrelated stresses, we cannot disregard the possibility that this striking competitiveness expressed by ATR+ rhizobia at low pH is a consequence of the enhancement of more general capabilities to face rhizospheric stresses. Note that ATR+ rhizobia were also slightly more competitive during the nodulation at pH 7.0 (Fig. 3b).

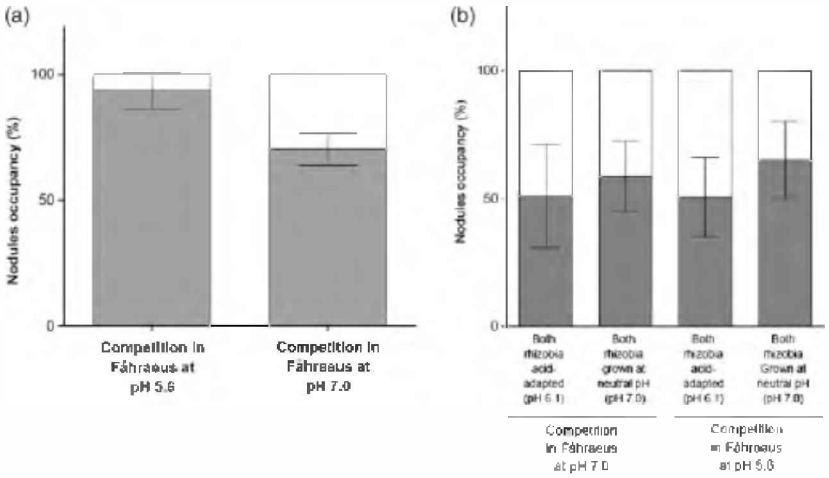

Fig. 3. Competition experiments for nodulation of alfalfa at different pH through the use of different mixtures of isogenic ATR+ and/or ATR − *Sinorhizobium meliloti* as inoculants. (a) (ATR −) *S. meliloti* 2011 (white bars), and its isogenic acid-adapted derivative (ATR+) *S. meliloti* MP6 (gray bars) were coinoculated onto alfalfa plants grown in Fåhraeus medium at either pH 5.6 or 7.0, as indicated. The rhizobia had been previously grown in Evans minimal medium at either pH 6.1 (for the ATR+) or 7.0 (for the ATR −) to an $OD_{600\,nm} = 0.2$, diluted in Fåhraeus medium, and then mixed together at a final concentration of 10^5 CFU mL^{-1} each. Fifty milliliters of the inoculant mixture were added to plastic pots with alfalfa plants and vermiculite at pH 5.6 and 7.0 as indicated in Materials and methods. The proportion of nodule occupancy by each rhizobium was analyzed at 30 days postinoculation. Because of the low proportion of co-occupancy of nodules in alfalfa (Lagares *et al.*, 1992), nonfluorescent nodules were scored for the ATR − rhizobia and the fluorescent ones for the ATR+ strain. A total of about 600 nodules were analyzed. (b) Control experiment where both rhizobia *S. meliloti* 2011 and *S. meliloti* MP6 were cultured in batch in parallel under the same pH condition (either both at pH 7.0 or both at pH 6.1) to result in either an ATR − or an ATR+ state, respectively. The pH during the precultivation of the rhizobia and during the nodulation in Fåhraeus medium are indicated for each experiment. The inoculation and analysis of the results were performed as indicated for the experiments in (a). The error bars indicate SDs.

In this study, we have shown that the entrance of *S. meliloti* into the adaptive ATR occurs under batch cultivation at moderately acid pH, but not in chemostat growth under continuous cultivation at the same acid pH, an observation that prompted us to question whether or not hydrogen ions themselves were the exclusive inducers of the transient state of acid tolerance. Although the same Evans medium was used in both experimental protocols, batch and continuous cultivation represent completely different growth systems: i.e. while a nutritional limitation must be present during the steady state in all continuous systems (N in this instance), the same limitations are not reached during the log phase of batch cultures. Our observations reveal the adaptive ATR as a complex process, triggered by an increased hydrogen-ion concentration, but operative only if other – as yet unknown – concomitant changes that depend on the cultural conditions are present. At the moment, in *S. medicae*, only the genes *actSR* have been reported to be necessary for the induction of the adaptive ATR (Glenn *et al.*, 1999). A careful analysis of target genes regulated by this two-component system might shed light on the conditions required, along with the cellular processes that need to be activated, for the ATR in *S. meliloti* to take place.

Although the stability and influence of the ATR on acid tolerance has already been characterized in rhizobia (O'Hara & Glenn, 1994; Dilworth *et al.*, 1999), no data were available at that time on the effect of the adapted state on symbiosis. To this end, we have shown here that the ATR confers a clear advantage on the rhizobia in the nodulation of the host roots under acidic conditions. From the practical point of view, the results presented here open a new avenue toward the possibility of using acid-adapted (ATR+) rhizobia as inoculants for acidic soils. Such a possibility would point to a consideration of such adapted rhizobia as candidates for an economically sound and biosafe alternative for the improvement of legume inoculation under acidic stress. It will certainly require new experiments with microcosms, and especially in the open field, to evaluate the performance of ATR+ rhizobia within natural soil environments. In addition, new investigations will be necessary that should be aimed at characterizing the appropriate conditions for stabilizing the positive symbiotic properties of ATR+ rhizobia in long-lasting inoculant formulations.

Acknowledgements

This investigation was supported by grants PIP5701, PICT14562, and PICT31937 to A.L.; and PICT2003-32915, PICT2006-404, and PIP2009-2474 to M.F.D.P., M.P. M.F.D.P., M.P., E.J., and A.L. are members of the Research Career of CONICET. The authors are grateful to Dr Donald F. Haggerty for editing the manuscript.

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